JC14 Rec'd PCT/PTO 16 JAN 2002

Form PTO-1390 (REV 10-2000) U.S.DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 1581.0890000/RWE/MTT

U.S. APPLICATION NO. (IF KNOWN, SEE 37 C.F.R. § 1.5): *To be assigned* 10/031082

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/02738	17 July 2000	16 July 1999

TITLE OF INVENTION: Storage of Microorganisms, Cells and Tissue

APPLICANT(S) FOR DO/EO/US: CODD, Anthony Arthur

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 372(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98 with 2 pages of Form PTO-1449 and 14 cited documents.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    a) Application Data Sheet;
    b) Authorization to Treat A Reply As Incorporating An Extension Of Time Under 37 C.F.R § 1.136(a)(3) *(in duplicate)*; and
    c) A copy of published International Application No. PCT/GB00/02738 including 26 pages of description prior to the claims, and 6 pages of claims (claims 1-38);
    d) Form PCT/ISA/210;
    e) Form PCT/IPEA/409 with annexes attached hereto;
    f) Form PCT/IPEA/416;
    g) Two (2) Return Postcards.

JC13 Rec'd PCT/PTO 16 JAN 2002

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
To be assigned 10/031082	PCT/GB00/02738	1581.0890000/RWE/MTT

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... **$ 100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	36 - 20 =	16	X **$18.00**	$ 288.00	
Independent Claims	1 - 3 =	0	X **$84.00**	$ 00.00	
Multiple dependent claim(s) (if applicable)			+ **$280.00**	$ 00.00	
TOTAL OF ABOVE CALCULATIONS =				$1308.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$	
SUBTOTAL =				$1308.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$1308.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$1308.00	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $ 1308.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
1100 New York Avenue, NW, Suite 600
Washington, D.C. 20005-3934

Robert W. Esmond 1-16-02
SIGNATURE DATE

Robert W. Esmond
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32,893
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CORRESPONDENCE INFORMATION

Correspondence Customer Number:: 26111
Fax One:: 202-371-2540

APPLICATION INFORMATION

Title Line One:: Storage of Microorganisms, Cells and Tis
Title Line Two:: sue
Total Drawing Sheets:: 0
Formal Drawings?:: No
Docket Number:: 1581.0890000
Secrecy Order in Parent Appl.?:: No

CONTINUITY INFORMATION

This application is a:: 371 OF
> Application One:: PCT/GB00/02738
Filing Date:: 07-17-2000

PRIOR FOREIGN APPLICATIONS

Foreign Application One:: 9916790.0
Filing Date:: 07-16-1999
Country:: Great Britain
Priority Claimed:: Yes

Source:: PrintEFS Version 1.0.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: *To be assigned*
CODD, A. A.	Art Unit: *To be assigned*
Appl. No. (U.S. Natl. Phase of PCT/GB00/02738)	Examiner: *To be assigned*
Filed: (Int. Filing Date: July 17, 2000)	Atty. Docket: 1581.0890000/RWE/MTT
For: **Storage of Microorganisms, Cells and Tissue**	

Preliminary Amendment

Commissioner for Patents
Washington, D.C. 20231

Sir:

In advance of prosecution of the captioned application, Applicants submit the following Preliminary Amendments and Remarks. This Preliminary Amendment is provided in the following format:

(A) A clean version of each replacement paragraph/section/claim along with clear instructions for entry;

(B) Starting on a separate page, appropriate remarks and arguments. 37 C.F.R. § 1.121 and MPEP 714; and

(C) Starting on a separate page, a marked-up version entitled: "Version with markings to show changes made."

It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R.

§ 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Amendments

Please amend the application as follows:

In the Specification:

In the specification at page 1, before line 1, please insert the following paragraph:

The present application is a 371 of PCT/GB00/02738 filed on July 17, 2000, and published in English on January 25, 2001.

In the Abstract:

Please insert following abstract on page 33 after the claims:

A composition for preserving viable microorganisms, cells or tissue comprises (a) a preservative combination of (i) a non-reducing disaccharide and (ii) a bulking agent; and (b) a buffer. Also described is a method of preserving viable microorganisms, cells or tissue, comprising combining viable microorganisms, cells or tissue with a preserving solution comprising a non-reducing disaccharide, drying the combination to form a dried preparation having a solids content of at least 80% by weight, and counting the viable microorganisms, cells or tissue in the dried preparation, whereby the dried preparation can be combined with aqueous buffer to yield an aqueous preparation comprising a predetermined count of viable microorganisms, cells or tissue.

This abstract is provided on a separate sheet appended hereto.

In the Claims:

Please amend the following claims from the currently pending claims as submitted under Article 34 to the International Bureau on July 30, 2001, and attached as annexes to the International Preliminary Examination Report:

Please cancel claims 2-34 without prejudice to or disclaimer of the subject matter contained therein. Applicants reserve the right to prosecute these claims in later continuing applications.

1. (Reiterated) A composition for preserving viable microorganisms, cells or tissue, the composition comprising:

(a) a preservative combination of (i) trehalose (ii) a matrix protein bulking agent and (iii) a monosaccharide; and

(b) a buffer.

Please add the following new claims:

35. (New) The composition of Claim 1 wherein the weight ratio of said trehalose to bulking agent is 5:1 to 0.5:1.

36. (New) The composition of Claim 1 wherein the weight ratio of said trehalose to bulking agent is 3.5:1 to 1.5:1.

37. (New) The composition of Claim 1 wherein said bulking agent is, or comprises, a protein of molecular weight 50-100KD.

38. (New) The composition of Claim 37 wherein said bulking agent is an albumin.

39. (New) The composition of Claim 1, comprising:

(i) 5 to 30 wt% trehalose;

(ii) 1 to 10 wt% bulking agent; and

(iii) 60 to 94 wt% aqueous buffer.

40. (New) The composition of Claim 1 wherein said monosaccharide is a reducing sugar.

41. (New) The composition of Claim 40 wherein said reducing sugar is glucose.

42. (New) The composition of Claim 41 wherein said glucose comprises about 0.1 wt% to about 3 wt% glucose.

43. (New) The composition of Claim 39, further comprising a structural additive.

44. (New) The composition of Claim 43 wherein said structural additive is water-soluble.

45. (New) The composition of Claim 44 wherein said structural additive is, or comprises, a water-soluble polymeric carbohydrate.

46. (New) The composition of Claim 43 wherein said structural additive is carboxymethylcellulose or hydroxyalkylcellulose.

47. (New) The composition of Claim 43 wherein said structural additive and all other components are water-soluble so that when a dried composition of the invention is reconstituted in water it dissolves substantially completely leaving a clear solution.

48. (New) The composition of Claim 1 further comprising a colouring agent.

49. (New) The composition of Claim 1 wherein said microorganisms are selected from the group consisting of bacteria, viruses, protozoa and fungi.

50. (New) The composition of Claim 1 wherein the solids content of said composition is in the range of from about 10 wt% to about 50 wt%.

51. (New) A process for preserving viable microorganisms, cells or tissue, comprising the steps of:

(a) combining the microorganisms, cells or tissue with the composition of Claim 1 to form a preserving preparation;

(b) drying the preserving preparation at a drying temperature not below 0°C; and

(c) storing the dried preparation obtained.

52. (New) The process of Claim 51 wherein step (b) is carried out at atmospheric pressure.

53. (New) The process of Claim 51 wherein step (b) is carried out by passing a draught of air, inert gas or a mixture thereof over the preserving preparation in the presence of a drying agent.

54. (New) The process of Claim 53 wherein step (b) further comprises:

(i) partially drying the preserving preparation by passing a draught of air, inert gas or a mixture thereof over the preserving preparation in the presence of a drying agent; and

(ii) subjecting the partially dried preserving preparation to further drying at reduced temperature.

55. (New) The process of Claim 53 wherein said drying agent is silica gel.

56. (New) The process of Claim 51 comprising drying the preserving preparation at reduced pressure.

57. (New) The process of Claim 51 comprising depositing the preserving preparation onto a hydrophobic surface prior to drying.

58. (New) The process of Claim 57 wherein said volume of preserving preparation deposited onto the hydrophobic surface is in the range of from about 5 µl to about 100 µl.

59. (New) The process of Claim 51 wherein one or more of the steps (a) to (c) are carried out in a substantially oxygen-free environment.

60. (New) A dried composition according to Claim 1 further comprising a viable microorganism and having a solids content of at least about 80%.

61. (New) The dried composition of Claim 60 wherein said viable microorganisms are bacteria, or a mixture of bacteria of different strains, or a combination of bacteria and mammalian cells.

62. (New) The dried composition of Claim 60 having a defined microorganism count wherein said microorganism count remains substantially stable in storage at ambient temperature.

63. (New) The dried composition of Claim 60 wherein the composition is fully dispersible when reconstituted with water or aqueous buffer solution.

64. (New) A method of preserving viable microorganisms, cells or tissue, comprising:

(a) combining viable microorganisms, cells or tissue with a preserving solution comprising the composition of Claim 1;

(b) drying the combination of (a) to form a dried preparation having a solids content of at least 80% by weight; and

(c) counting the viable microorganisms, cells or tissue in the dried preparation;

whereby the dried preparation can be combined with aqueous buffer to yield an aqueous preparation comprising a predetermined count of viable microorganisms, cells or tissue.

65. (New) The method of Claim 64 wherein said method comprises forming at least first and second dried preparations, counting the viable microorganisms, cells or tissue in the first dried preparation and thereby estimating the number of viable microorganisms, cells or tissue in the second dried preparation.

66. (New) The method of Claim 65 comprising forming a plurality of dried preparations, and counting a selected sample of those dried preparations so as to estimate the count of viable microorganisms, cells or tissue in the remaining dried preparations.

67. (New) A method of preserving viable microorganisms, comprising drying an aqueous preparation of viable microorganisms in the presence of the composition of Claim 1 to form a dried preparation, counting the viable microorganisms in the dried preparation to obtain a counted dried preparation, and storing the counted dried

preparation, wherein the counted dried preparation when reconstituted with water or aqueous medium forms an aqueous preparation containing a predetermined number of viable microorganisms.

68. (New) The method of Claim 67 wherein said method comprises forming at least first and second dried preparations, counting the viable microorganisms, cells or tissue in the first dried preparation and thereby estimating the number of viable microorganisms, cells or tissue in the second dried preparation.

69. (New) The method of Claim 68 comprising forming a plurality of dried preparations, and counting a selected sample of those dried preparations so as to estimate the count of viable microorganisms, cells or tissue in the remaining dried preparations.

Remarks

By the foregoing amendments, claims 2-34 have been cancelled and new claims 35-69 are sought to be entered. Support for the foregoing amendments to the claims may be found throughout the specification and in the claims as originally filed. Accordingly, the present amendments do not add new matter, and their entry is respectfully requested. Upon entry of the foregoing amendments, claims 1 and 35-69 are pending in the application, with claim 1 being the independent claim.

It is believed that the present application is in condition for immediate examination. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Robert W. Esmond

Robert W. Esmond
Attorney for Applicants
Registration No. 32,893

Date: Jan. 16, 2002

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SKGF Rev. 3/16/01

Version with markings to show changes made

In the Specification:

In the specification at page 1, before line 1, please insert the following paragraph:

The present application is a 371 of PCT/GB00/02738 filed on July 17,2000, and published in English on January 25, 2001.

In the Abstract:

Please insert following abstract on page 33 after the claims:

A composition for preserving viable microorganisms, cells or tissue comprises (a) a preservative combination of (i) a non-reducing disaccharide and (ii) a bulking agent; and (b) a buffer. Also described is a method of preserving viable microorganisms, cells or tissue, comprising combining viable microorganisms, cells or tissue with a preserving solution comprising a non-reducing disaccharide, drying the combination to form a dried preparation having a solids content of at least 80% by weight, and counting the viable microorganisms, cells or tissue in the dried preparation, whereby the dried preparation can be combined with aqueous buffer to yield an aqueous preparation comprising a predetermined count of viable microorganisms, cells or tissue.

In the Claims:

Claims 2-34 have been cancelled.

New claims 35-69 are sought to be entered.

STORAGE OF MICROORGANISMS, CELLS AND TISSUE

ABSTRACT

A composition for preserving viable microorganisms, cells or tissue comprises (a) a preservative combination of (i) a non-reducing disaccharide and (ii) a bulking agent; and (b) a buffer. Also described is a method of preserving viable microorganisms, cells or tissue, comprising combining viable microorganisms, cells or tissue with a preserving solution comprising a non-reducing disaccharide, drying the combination to form a dried preparation having a solids content of at least 80% by weight, and counting the viable microorganisms, cells or tissue in the dried preparation, whereby the dried preparation can be combined with aqueous buffer to yield an aqueous preparation comprising a predetermined count of viable microorganisms, cells or tissue.

STORAGE OF MICROORGANISMS, CELLS AND TISSUE

This invention relates to the storage of microorganisms, cells and tissue. The invention may be applied to the storage of a wide range of such biological materials but in particular has a special applicability to storing those biological materials that deteriorate at room temperature in aqueous solution. The invention in its most preferred aspect relates to the storage of microorganisms, cells and tissue in a viable state.

Many varieties of biological reagent are known to deteriorate rapidly when stored at room temperature, forcing the adoption of a range of methods for the storage and preservation of such reagents. Known methods include simple refrigeration, and freeze-drying and subsequent storage at ambient temperature. In some instances, no effective storage methods as available. Instead, an assessment is made of the deterioration of the sample between its preparation and analysis so that the original composition of the sample can be extrapolated.

A method of preserving microorganisms (including bacteria, yeast and fungi) is known that uses storage beads. A suspension of the microorganism is made in a cryopreservative and then mixed with storage beads. The cryopreservative is removed and the beads frozen and stored at a temperature of typically -70°C. To recover the microorganism, a bead is removed from frozen storage and placed in broth or rolled onto growth medium. This method has various drawbacks. Storage temperatures of 70°C require specialist refrigeration units. Freezing inevitable results in significant physical damage to the microorganism. Special cryopreservative is required, which can be expensive. Finally, the beads are typically of size 2-4mm diameter and are awkward to manipulate.

In some known instances it is required to obtain a viable count from a water sample that possibly contains bacteria. At present, this is routinely achieved

by transport of the test sample in solution from the place at which the test sample was obtained to the counting location. It is a known phenomenon that, during transport, the number of viable bacteria in the sample will decrease over time at a certain rate. To counter this, the bacterial count is taken at a very precise and predetermined time interval after the sample is taken. The count is then adjusted according to the calculated decrease in number since the sample was taken. Disadvantages inherent in this existing method include the danger of rupture of the vessel that contains the bacterial sample and the inconvenience of being forced to operate within a precise time scale intended to allow for the known rapid deterioration in viability of the bacterial sample over time.

EP-A-0229810 describes protection of proteins and like macromolecules using a solution of trehalose. However, the particular difficulties associated with storage of microorganisms is not addressed.

It is widely recognized that food safety is highly dependent on bacteriological methods that are capable of detecting small numbers of pathogenic bacteria in both raw and processed materials. Contamination below an infecting dose given favourable conditions for growth such that re-hydration of a dry product or exposure of a wet product to a high ambient temperature may soon become hazardous.

One of the problems of controlling the performance of routine microbiological tests on foods for the presence or absence of marker organisms is the provision of suitable standardized controls. Many laboratories rely on wet suspensions cryo-preserved on beads at -20°C for a number of weeks or broths refrigerated at 4°C and renewed weekly. The inocula are prepared by making a fixed dilution from the preserved suspensions but the actual counts obtained can vary widely depending on the density and viability of the organism. Freshly grown bacteria are usually less damaged than cells that have been resting for a while and will consequently have a higher

plating efficiency. However marker organism of interest to public health and the food industry are often in a non-replicating and damaged state. In addition they make have been subjected to thermal stress during the manufacturing process.

US-A-5733774 describes methods and compositions for producing stable bacterial formulations, and involves drying bacteria, combining them with a powder or granular non-aqueous carrier and packaging the bacteria in sealed packaging impermeable to gas and water vapour, the method also including removing substantially all oxygen from inside the package. The methods described, however, are impractical in that strenuous efforts must be taken to remove gas and water vapour and oxygen from the stored material. In addition, prolonged viability of stored bacteria has not been demonstrated.

WO-A-90/04329 describes storing mammalian cells in a process that includes lyophilizing mammalian cells and storage at about 4°C. The lyophilizing step introduces significant risk of loss of viability of the stored material.

WO-A-99/00485 discloses a composition for preserving microorganisms, using starch optionally mixed with albumin.

The present invention seeks to overcome or at least mitigate problems associated with the art and to provide improved storage of biological material such as microorganisms, cells and tissue.

It is therefore an object of the present invention to provide a method of storage of biological materials that does not require refrigeration below 0°C and does not require freezing of the stored material.

It is another object to provide a solid-state stored biological material that is stable to an acceptable degree and suitable for long-term storage at 4°C and

which can readily be re-constituted into a solution of biological material.

A further object is to provide a stored biological material which is suitable for transport, including via the postal system, without excessive risk of damage to the biological material during transport.

A more specific object of the invention is to provide a method of storing microorganisms, cells and tissue in a viable state for extended periods of time.

Accordingly, in a first aspect, the invention provides a method of preserving viable microorganisms, comprising drying an aqueous preparation of viable microorganisms, cells or tissue in the presence of non-reducing disaccharide to form a dried preparation, counting the viable microorganisms, cells or tissue in the dried preparation to obtain a counted dried preparation, and storing the counted dried preparation, wherein the counted dried preparation when reconstituted with water or aqueous medium forms an aqueous preparation containing a predetermined number of viable microorganisms, cells or tissue.

In a second aspect, the invention provides a composition for preserving viable microorganisms, cells or tissue, the composition comprising:

(a) a preservative combination of (i) a non-reducing disaccharide and (ii) a bulking agent; and

(b) a buffer.

In a third aspect, the invention provides a process for preserving viable microorganisms, cells or tissue, comprising the steps of:

(a) combining the microorganisms, cells or tissue with the composition of the invention to form a preserving preparation;

(b) drying the preserving preparation at above the triple point of water; and

(d) storing the dried preparation obtained.

It is an advantage of the invention that biological material such as microorganisms, cells or tissue can be stored for extended periods of time in a stable form and whilst retaining significant viability. Specific embodiments of the invention provide individual dried preparations of bacteria suitable for storage and which are readily reconstituted with water or other aqueous buffer to yield viable bacteria. Thus, a 25μl drop of bacterial suspension forms a lenticule with a dry weight of 8mg, containing from 10-10^8 CFU's of single organisms or mixtures. It can be reconstituted for use in 10 minutes.

A further advantage of the invention over other methods of preserving viability in microorganisms is that samples of biological material can be prepared having a predetermined, known number of viable microorganisms, cells or tissue, due to the extended viability of stored material in compositions of the inventions. Samples can thus have a defined numbers of viable organisms per stored sample. Hence, they can be used for quantitative experiments, and in situations where presence of absence of very low-numbers of marker organisms is a stringent test of the processes being controlled.

Defined count preparations containing bacterial numbers ranging from 10 cfu to 10^8 cfu have been prepared in accordance with the invention, stored and resuscitated with no significant loss of viability. The confidence limits of the cfu counts have been established by performing viable counts on multiple lenticules. The counts are usually within 95% confidence interval, even for the very low count preparations. The invention provides a versatile alternative to the maintenance of bacterial cultures either on slopes, wet suspensions at 4°C, cryo-preservation at low temperature, freeze-drying or spray-drying.

In use of the invention, a sample of biological material is combined with (a) a preservative combination of (i) a non-reducing disaccharide and (ii) a bulking agent; and (b) a buffer. The weight ratio of the non-reducing disaccharide:bulking agent is suitably 5:1 to 0.5:1, preferably about 3.5:1 to 1.5:1, and the solids content is generally at least 20% by weight.

The non-reducing disaccharide may be selected from the group consisting of trehalose, sucrose, maltose, lactose, cellobiose, isomers thereof and mixtures thereof. Preferably, trehalose or a combination of trehalose and other non-reducing disaccharide is used.

In compositions of the invention, the bulking agent typically is, or comprises, a high molecular weight protein. Preferably the bulking agent is a matrix protein which has a molecular weight which is less than 100kD and preferably is within the range 50-100kD. Most preferably the matrix protein is a relatively inert globular protein such as albumin. Gelatin, (which normally has a molecular weight in the range 170-200kD) is not suitable. Suitable albumins include ovalbumin, foetal calf albumin and lactalbumin. In an embodiment of the invention the matrix protein is serum albumin, or more specifically bovine serum albumin. The matrix protein is conveniently present in solution in an amount between 5 and 20g per 100ml, preferably 8-12g and more preferable around 10g per 100ml.

The buffer is selected so as to maintain the pH of the solution at a value at which the microorganisms, cells or tissues are stable and should contain components that do not selectably salt out as their concentration increases during the drying step. One suitable buffer is phosphate buffered saline (PBS) at a concentration between 0.1 and 1M, preferably 0.15-0.5M.

The above components act together to form a solution which when dried results in a solid, typically flexible, pellet containing the biological material that is being stored.

A particular embodiment of the invention comprises:

(i) 5 to 30 wt% non-reducing disaccharide;

(ii) 1 to 10 wt% bulking agent; and

(iii) 60 to 94 wt% aqueous buffer.

The composition may further comprise a monosaccharide, for example a reducing sugar such as glucose. The monosaccharide can be present at 0.1 to 3 wt%.

It is further optional to include within the composition a structural additive, and this can be or comprise a water-soluble polymeric carbohydrate. In embodiments of the invention carboxymethylcellulose is used, though other additives are suitable, such as hydroxyalkylcellulose.

It is preferred that the structural additive be water soluble and that all other components be water soluble so that when a dried composition of the invention is reconstituted in water it dissolves substantially completely leaving a clear solution. Insoluble or partially soluble sugars and bulking are preferably avoided. Thus, starch which does not fully dissolve or dissolves leaving a cloudy preparation is preferably avoided.

A colouring agent is further optional.

The methods and compositions of the invention are particularly suited to storage of microorganisms, selected from the group consisting of bacteria, viruses, protozoa and fungi.

The solids content of compositions of the invention is initially generally in the range of from 10-50%.

Once biological material has been combined with the composition of the invention, it is preserved by drying at above the triple point of water, and

storing the dried preparation obtained. The drying step can readily be carried at atmospheric pressure, and good results have been so obtained in examples of the invention described in more detail below. Reduced pressure can also be used in drying the compositions.

Drying may also be carried out by passing a draught of air, inert gas or a mixture thereof over the preserving preparation in the presence of a drying agent. Silica gel can be used, as can other drying agents. It is also optional to use a substantially oxygen-free environment.

In a further embodiment of the invention, the process comprises:

(i) partially drying the preserving preparation by passing a draught of air, inert gas or a mixture thereof over the preserving preparation in the presence of a drying agent; and

(ii) subjecting the partially dried preserving preparation to further drying at reduced temperature.

In a particular embodiment of the invention, the process comprises depositing the preserving preparation onto a hydrophobic surface prior to drying. Preferably, a small volume of liquid is deposited and dried, forming a small solid composition, referred to as a pellet or lenticule. Once dried this pellet is highly convenient to handle. The volume of preserving preparation that constitutes a small volume can be in the range of from 5-100 μl.

In a specific embodiment, the process of the invention as applied to bacteria is to heavily seed a supportive medium with a culture and incubate it optimally until early lag phase. On a standard 90mm plate the typical yield is 10^{11} colony forming units, CFU's. This growth is harvested into a small volume of low strength saline and mixed with 2.5ml of solution. 25μl spots are dispensed onto a hydrophobic surface and dried above the triple point. The 100 or so resulting primary so-called lenticules each contain around 10^8 CFU's and are stored at about -20°C, above the eutectic, under desiccation.

Strains that have been preserved by the invention include *Staph. aureus, Staph. epidermidis, Bacillus cereus, Bacillus subtilis, Listeria monocytogenes, Str. faecalis, Str. pyrogenes, Str. agalactia, Str. pneumonia, Enterococcus faecium, Cl. perfingens, Sacchromyces, E coli (including O157), Salmonella sp, Ps aeruginosa, Klebsiella sp, Proteus sp, Campylobacter sp, Helicobacter pylori, Vibrio parahaemolyticus, Acenitobacter sp, Serratia marcesans, Haemophilus influenzae, Bordetella pertussis, N. menigitidis, N. gonorrhea, Bacteroides sp* and *Salmonella gold coast*. Mycoplasma, viruses and fungi can also be preserved by the present invention.

Compositions of the invention may be supplied in a number of formats, e.g

(1) on parafilm or similar hydrophobic surface;

(2) loose; and

(3) dried directly in microtiter strips, for all of these, storage at -20°C under desiccation assists a prolonged shelf life.

In a further aspect of the invention there is provided a dried composition according to the invention further comprising a viable microorganism and having a solids content of at least 80%. The viable microorganisms are preferably bacteria, or a mixture of bacteria of different strains, or a combination of bacteria and mammalian cells.

This aspect of the invention also provides a dried composition having a defined microorganism count wherein said microorganism count remains substantially stable in storage at ambient temperature. By defined count is meant that the content of the dried composition is known or predetermined, directly or indirectly, and that therefore once reconstituted with buffer an aqueous preparation is obtained having a defined count of viable microorganisms.

It is preferred that the composition is fully dispersible when reconstituted with water or aqueous buffer solution - ie that all components are water

soluble and readily dispersible.

A method of preserving viable microorganisms, cells or tissue of the invention comprises:-

(a) combining viable microorganisms, cells or tissue with a preserving solution comprising a non-reducing disaccharide;

(b) drying the combination of (a) to form a dried preparation having a solids content of at least 80% by weight; and

(c) counting the viable microorganisms, cells or tissue in the dried preparation;

whereby the dried preparation can be combined with aqueous buffer to yield an aqueous preparation comprising a predetermined count of viable microorganisms, cells or tissue.

To calculate the viable count it is preferred to form at least first and second dried preparations, counting the viable microorganisms, cells or tissue in the first dried preparation and thereby estimate the number of viable microorganisms. cells or tissue in the second dried preparation. Thus the count is made indirectly.

Where a large number of such dried preparations are made, the method can comprise counting a selected sample of those dried preparations so as to estimate the count of viable microorganisms, cells or tissue in the remaining dried preparations.

The drying step can be carried out in air or inert gas or mixtures thereof. In an embodiment, the drying step is carried out in an atmosphere of reduced oxygen concentration (compared to air), an advantage of this being that

oxidation of the biological material during the drying step is reduced. In another embodiment, the drying step is carried out in an atmosphere of reduced humidity. In a further embodiment, the pressure is reduced during drying.

The drying can conveniently be carried out at room temperature and can also be carried out at temperatures down to around 4°C. Higher drying temperatures could also be employed, though these would have to be weighed against the increased deterioration of the biological material caused by the increased temperature.

The drying step is intended to result in a solid composition containing the biological material and preferably having a water content in the region of less than 10 percent by weight. The water content is preferably in the range of 4-8 percent by weight and most preferably around 6 percent by weight (the term "around" allowing a tolerance of +/- 2%). As will be appreciated, the ideal water content of the solid stored composition will depend upon the nature of the material to be stored.

After the method has been used to produce a solid composition containing the biological material this then can be stored either at room temperature or, conveniently, at refrigeration temperature, typically around 4°C.

To re-constitute active biological material from the stored solid it is a fairly straight forward procedure to dissolve the stored solid in water or in buffer solution.

In embodiments of the invention the storage method is carried out under sterile conditions. Similarly, in embodiments of the invention the re-constitution of aqueous solution of biological material is carried out using sterile water or sterile buffer.

The invention is of application to biological material that cannot be stored indefinitely at room temperature in aqueous solution. A biological material may be regarded as having an unacceptable stability if it has a half life of less than 5 days, especially less than 2 days when stored at ambient temperature (20°C). Especially unstable biological material are ones having a half life of less than 24 hours. Examples of biological material that are suitable for storage according to the invention include microorganisms including bacteria and viruses, cells and samples of biological solutions such as serum samples.

In an embodiment of the invention, the storage method is used to store a bacterial sample. For storage of bacteria it is optional to include in the storage solution, prior to drying, a membrane stabilizing agent. Suitable membrane stabilizing agents include glycerol, egg yolk and mixtures thereof. It is particularly advantageous to store microorganisms such as bacteria using the method of the invention as when the bacteria are in solid, stored form they are easy to handle and transport, including transport via the postal service. The bacterial in the stored form of the invention are relatively stable and this enables the viable count to be obtained over a more flexible time scale than previously possible.

In a preferred embodiment of the invention the bacterial storage method includes providing a nutrient medium for the stored bacteria.

A first solution may be prepared according to the invention containing a matrix protein and a mono-or di-saccharide in 100ml of buffer. It is optional to add a small amount, in the range of 0.01-1g of peptone.

A second solution may be prepared acting as a nutrient medium for the bacteria. This nutrient medium will routinely include essential nutrients for growth/survival of the bacteria. In addition, it may optionally also include bacterial membrane stabilizing components. The bacteria are grown/cultured

and then pelleted and mixed with a small volume of nutrient medium. A small volume of (1) the nutrient medium containing the viable bacteria is mixed with (2) a larger volume of the storage solution and subjected to drying as before to give a residual solid with water content approximately 4-8 percent by weight.

To re-constitute the viable bacterial sample the solid is dissolved in water or buffer or nutrient solution.

In another embodiment of the invention, a preparation of the biological material to be stored (B) is mixed with a solution of the invention (S) in a ratio {of B/S} of between 1:5 and 1:10. Volumes of the mixed solution in an amount of between 10 and 50μl are dispensed onto a hydrophobic film, for example parafilm, or into microtitre strips. This results in small separated liquid volumes. At this stage the biological material is in a stabilizing solution in a droplet of given volume which contains a desired quantity of biological material. The separated volumes are then dried to give residual solid lenticules or pellets with a water content in the range 4-8 percent by weight, preferably around 6 percent by weight.

The process differs from freeze drying in that the drying temperature is not below 0°C nor the pressure significantly below atmospheric. The stabilizing solution typically contains an initial level of around 30 percent dissolved solids. As drying proceeds, the individual volumes becomes highly viscous and, when on the hydrophobic film, assume the shape of a plano-convex disk, i.e. lens like. As drying proceeds further, the discs become solid and glass-like. Although the size and weight of the solid pellets can vary over a wide range, the invention is particularly applicable to the production of these relatively small solid pellets.

Typical pellets produced according to the invention may weigh less than 1mg and their weights can be as 1-100mg or lower. It will be appreciated

that the final weight and size is determined by the volume and concentration of the solution before drying commences and the water content of the solid. Thus, for example, when the volume of the solution being dried is in the range 1-100μl, preferably 10-50μl and the solid content is around 33 wt%, the final dried weight will be around 0.33-33mg, preferably 3.3-16mg. The final dried solid discs are typically between 1-5mm in diameter. They are suitable thereafter for storage at 4°C or less, and preferable under desiccation, and will not deteriorate significantly at ambient temperature, e.g. if in transit. They can be stored at reduced temperature, down to -20°C or lower, in the presence of a dehydrating substance, e.g. silica gel.

The precise composition of the stabilizing solution will vary according to the materials being preserved. For example, many buffers may be appropriate, though it is essential that the components of the buffer do not selectively salt out as the concentration of the buffer increases as drying proceeds.

It will be appreciated that there is a degree of empiricism with the selection of the substances used in the stabilizing solution. The basic ingredients are designed to ensure that the solid material dries out into a glass like solid which is firm and free of crystal formations. Other additional components may optionally be added such as glycerol (membrane stabilizing), ascorbic acid (reducing agent), peptone, amino acids, calcium, magnesium and phosphate ions, charcoal and soluble starch. These additional substances are typically present in amounts between 0.01 and 1 percent weight by volume. The additional components are added to act as plasticizers, reducing agents, nutrients, membrane protectors and anti-oxidants.

Preferably the composition contains water in an amount of between 4-8% by weight of the total composition.

A marker dye is optional, functioning only for convenience in identifying the stored material. The same solution and method can be used for the storage

of conjugated antibody.

There is sometimes a marked reduction in the viable counts of some organisms, such as Aeromonas, during drying. Allowance must be made for this, but once dehydrated, no further increased loss of viability occurs.

Example 1

Storage Solution Formula

A solution is prepared having the following composition:-

Trehalose	240 g
Bovine serum albumin (filtered)	80 g
Glucose	10 g
CMC (BDH, low viscosity)	20 g
Pages saline (Oxoid)	1 litre

The BSA is analyzed and contains only 5 milli-equivalents of sodium. Pages saline is used because it stabilises biological entities. It is a dilute saline containing low concentrations of sodium, magnesium, potassium and phosphorus. Other buffers may also be used. EC-approved food colourings are added on occasion. All have been tested at high concentrations against various bacteria and had no deleterious effect on viability.

Once it has been made up, the solution is autoclaved prior to use. Note that the concentrations of the ingredients will increase during dehydration. If a 25μl drop dehydrates to *c.* 7mg i.e. *c.* 7μl, everything is concentrated by a factor of 3.

Example 2

Dehydration of the Storage Solution

A storage solution from Example 1 is subjected to forced drying under a draught of filtered air in a drying cabinet containing silica gel for a few hours (3-4 hours for individual lenticules on Parafilm strips; overnight when dispensed into the wells of plastic strips). Thereafter, they are transferred to sealed plastic boxes containing silica gel and left in the fridge at 4°C to complete the dehydration process, which may take 7 days. When they have become "hard", which seems to be judged by eye, they are transferred to screw-capped plastic jars containing silica gel and stored at -20°C. Samples are removed for viable count testing after 7 days and at 3-6 monthly intervals thereafter.

Drying the lenticules under reduced pressure is an option. The silica gel, in the base of the cabinet, is regenerated by heating it in a hot air oven until it is bright blue. Other desiccants may also be used.

Example 3

Preparation of bacteria for Solution.

Typically, the bacterial strain to be preserved is subcultured onto solid media to obtain heavy growth i.e. *c.* 10^{11} colony forming units (cfu). After appropriate incubation (usually overnight at 37° C, such that the bacteria should be in the early lag phase of their growth cycle) the colonial growth is collected in a sterile loop, which holds *c.* 100 mg of material. This collected material is suspended in *c.* 0.5ml Pages saline, which is then added to 2.5 ml of lenticulating solution and mixed very thoroughly to avoid clumping. Satisfactory mixing can be achieved by sucking a small volume up and down in a fine-tipped pipette.

Once an even suspension has been achieved, the inoculated fluid is dispensed in drops weighing *c.* 1mg onto a hydrophobic surface such as Parafilm, stretched taut over a flat sheet of material such as Perspex. There should be sufficient for 100 such spots, each containing *c.* 10^{8} cfu.

In order to make defined-count lenticules the inoculum can be factored down from these high-count "stock" lenticules. A Miles and Misra viable count is performed, and a lenticule is rehydrated in maximum recovery diluent (MRD), diluted appropriately in MRD and then added to lenticulating solution at the required concentration i.e. the low-count lenticules are prepared from the stock lenticules without a culture step.

Example 4

Preparation of cells from urine for preservation by lenticulation

White blood cells and epithelial cells from human urine are stored to be used in making simulated clinical samples for QA purposes.

1 litre of urine is pooled from clinical samples known to have high cell counts. The red blood cells are removed by adding 20ml glacial acetic acid dropwise, with stirring. This also dissolves the phosphates. The urine is allowed to stand so that the remaining cells will settle out slowly. It can not be centrifuged at this stage because that would cause the cells to stick together.

After a few hours, most of the supernatant is decanted off and approximately 100ml of 0.1M disodium, dihydrogen phosphate buffer at neutral pH is added to neutralise the acetic acid. The suspension is allowed to stand again and most of the supernatant is decanted. Phosphate buffered saline (PBS) is added to restore the volume to *c.* 100ml. This is now a suspension of white blood cells, epithelial cells and a few bacteria. One part in 800 of glutaraldehyde is added to fix the cells, preserving their morphology. The cells are pelleted by centrifuging at a slow speed such that the bacteria remain in the supernatant. The cells are rinsed once in PBS and can then be stored at –4°C or cryopreserved by the addition of 30% glycerol and storing at –20°C. They can be added to the storage solution of Example 1 at the desired concentration, with or without bacteria or other biological

material, and dehydrated in the usual manner.

Example 5

Preparation of red blood cells for lenticulation

Sheep red blood cells are prepared for storage by fixing them in glutaraldehyde as in Example 4 and sedimenting them.

Example 6

Preparation of simulated faeces

Biodegradable plant material is dried, crushed and heat-treated with enteric pathogens to simulate faecal material for QA tests and stored using the solution of Example 1.

Example 7

Preparation of background flora for simulated throat swabs

Blood agar plate cultures of throat swabs are selected containing no pathogenic bacteria, but a wide variety of typical, non-pathogenic, mouth flora. The biomass is pooled from several plates and stored as in Examples 1-3. Thereafter, these could be rehydrated and added to fresh lenticulating solution at appropriate dilution to provide high, medium or low background counts. Suspensions of the target pathogen can be added, again at known concentrations, and the complex suspensions dispensed and dried as before. These can be used in National External Quality Assessment Schemes such as UKNEQAS.

Alternatively, the storage solution containing the background flora and pathogens is distributed and dehydrated in plastic cupules. The product is rehydrated with a defined volume of MRD. Standard cotton swabs are dipped into the suspension, as each will take up a standard volume, several swabs being tested in each laboratory receiving the QC sample, according to their standard operating procedure (SOP) for culturing throat swabs.

Example 8

Other microorganisms preserved by lenticulation.

Using the materials and methods of Examples 1-3, Cryptosporidium oocysts, certain fungi such as Candida, and Mycoplasma are stored. Mycoplasma are

cultured in broth for several days prior to storage. The cells are then spun out in a microfuge and resuspended in half the original volume in storage solution. The stored material is rehydrated on a solid medium and carefully spread over a circular area of *c.* 1cm radius. After several days' incubation, the surface of the plate is examined under a low-power microscope to view the colonies of Mycoplasma.

Viruses have been successfully stored, preserving their morphology for electron microscopy QA samples, and preserving viability in Influenza A and Adenoviruses for over a year.

Example 9

Rehydration of lenticules

Viable microorganisms are retrieved from the stored sample by rehydrating them. Typically, a stored sample can be placed on the surface of a suitably supportive solid medium such as blood agar, and allowed to rehydrate for 10 minutes. Thereafter, it can be spread with a sterile plastic loop over the surface of the plate in the usual way.

Alternatively, a stored sample can be added to a volume of a liquid, typically maximum recovery diluent (MRD) and allowed to dissolve completely. The liquid is then swirled to ensure even mixing and sampled as desired.

Example 10

Use of Preparation of the Invention in "spiking" dried foods for Food EQA Schemes.

Food EQA Schemes typical want to assess the ability to detect the presence or absence of very low counts (10 cfu in 25g dried food) of specific pathogens. Low-count preparations are particularly suited to this. Duplicate preparations each containing 10 cfu of the target organism (or not) are

supplied. One preparation is rehydrated directly onto solid media and a colony count performed. The other is added to 25g of dried food, which is then subjected to the laboratory process being controlled. One hundred per cent recovery is not expected, because of the presence of free radicals, toxins or competitor organisms in the food. A failure-to-detect rate of 5% is acceptable to the food industry, but ideally the reference material should be supplied close to the limit of detection to provide a sufficiently stringent test.

Thus, if more than 5% of the results from processed foods fail to correspond with those from the direct plating of the paired lenticules, further investigation of the processing is warranted. *Salmonella goldcoast* has been used in this work to date because it is a durable Salmonella, is monophasic and relatively uncommon.

To introduce such presence/absence lenticules into a Food EQA Scheme would be straightforward. The lenticule content could be dictated by the specific process to be controlled. The ability to produce up to 10,000 identical preparations at a time has the great advantage that replicate samples are available for any test laboratory experiencing difficulty with their food processes.

Example 11

Storage of simulated urine

Materials and Methods

STRAINS

Routine isolates from urinary tract infections were collected over a period of one month. These included E coli, "coliforms," Klebsiella sp., Pseudomonas and Str. faecalis. In addition we selected NCTC strains of Acinetobacter, Ps. fluorescens MRSA and C. albicans to extend the range of organisms for inclusion in the panel. Strains were chosen for their typical colonial appearances, plated onto a supportive medium, usually blood agar, and

incubated overnight or longer for maximal yield. These cultures were preserved by the method of Example 1 and stored at -20°C pending further tests. A proportion of the lenticules were held at room temperature, 30°C and 37°C for 7 days reconstituted and cultured. Strains showing a substantial fall in count at the higher temperatures were rejected on the premise that an accelerated degeneration test would predict their probable long term storage. Pus and epithelial cells from the urinary tract were collected by pooling a substantial number of fresh urine samples submitted for routine examination. To one litre of this pool 20ml of glacial acetic acid was added and quickly mixed. After standing at room temperature for 4 hours most of the cellular content had settled into the bottom 20% volume. The supernatant was decanted and the remaining 200ml centrifuged very lightly at 1,000 rpm, to deposit the cells. These were washed twice in PBS and cryo-preserved at -20°C as a 10% suspension until required. Group O positive red cells were also cryo-preserved as a 10% suspension at -20°C.

Urine specimens were made from the stored bacteria and the cryo-preserved suspensions of cells by reconstitution in storage solution at a predetermined level. These levels were set to resemble those of fresh urine samples and included counts above and below 10^5 CFU's either of single organisms or mixtures. In addition some samples contained pus cells or red cells or both at an easily observable concentration. The mixtures were dispensed into 8 well flat bottomed microtitre strips each position being occupied by a different sample. Four batches were made giving a total of 32 samples. However one sample was replicated in batches 2, 3 and 4. The set also included some completely negative samples. The batches of strips were assembled into plates, labelled and dried by forced ventilation in a desiccating cabinet at 20°C followed by standing at 4°C for 72 hours over silica gel to complete the process. The plates were stored at -20°C pending use.

MEDIA

All routine culture media was supplied by Oxoid in powder form which was prepared on site in accordance with the manufacturer's instructions. Columbia Agar Base CM331 with 5% horse blood from TCS for Blood Agar plates, CLED CM. Solutions were prepared as needed from stock reagents.

PROCEDURE FOR TESTING

Prior to use the strips were re-hydrated by the addition of 100μm of MRD to each well, left for 10 minutes and shaken to disperse the bacterial and cellular suspensions. These specimens were examined by the laboratory's standard operating procedure. Cells were observed with an inverted microscope with the specimens in situ. Care was needed to ensure that the strips were correctly orientated as the identification of each sample depended upon its position, 1-8, in the strip. Each specimen was spread using a standard 1μl loop onto half of a standard 90mm CLED plate and incubated overnight aerobically at 37°C. The plates were read, scored and recorded by the same individual that processed the specimen the previous day. Sensitivity tests and identifications were carried out on a some of the isolates.

All specimens in the four batches were tested independently at various times for over a year both by microscopy and culture to ascertain their keeping properties. To improve the accuracy of these tests the counts were done by Miles and Misra (1938, Journal of Hygiene, 38, pp 732-749) as the volumes picked up by the standard loop method vary considerably.

RESULTS

The results are shown in Table A.

TABLE A

Survival of Organisms in Simulated Urine, CFU's per ml

Sample	Organism	90 Days	180 Days	360 Days	35 Months
A	E Coli	2.5×10^4	6.5×10^4	3.6×10^4	7.2×10^4
B	E Coli	1.7×10^6	1.6×10^6	2.6×10^6	1.8×10^6
C	Str faecalis	4.9×10^5	8.5×10^5	8.5×10^5	8.0×10^5
D	Klebsiella	6.7×10^5	8.8×10^5	1.0×10^6	1.0×10^4
E	Pseudomonas	1.9×10^5	4.0×10^5	2.7×10^5	2.0×10^5
F	E Coli	6.5×10^4	8.7×10^4	1.0×10^5	5.3×10^4
G	Str faecalis	1.2×10^5	3.5×10^5	2.3×10^5	3.5×10^5

The results show no evidence of falling counts on preserved organisms over a period of one year or, with the single exception of the Klebsiella sample, 35 months. In addition the morphology of the cellular components of the samples remained unaltered.

Example 12

Storage of *E Coli*

Lenticules containing *E Coli* were prepared according to Example 3 and viable counts on blood agar or on storage solution taken at day 1, 90 and 220. The results are shown in table B.

Thus, the invention provides an efficient method of storing biological materials in solid form. The materials are acceptably stable in solid form and are easily reconstituted into working solution. Many practical applications of the invention are evident, such as storage of bacterial samples for transit.

TABLE B

E coli NCTC 12950 batch 980521 Counts on groups of 30 lenticules.

On Blood Agar (BA) Day 1	60	79	81	82	87	87	
	89	90	90	90	92	92	
	93	94	94	95	96	96	
	97	97	100	100	101	101	
	102	103	104	105	105	109	mean 94
On Example 1 Solution (ES) Day 1	48	50	53	53	54	54	
	55	55	56	56	56	57	
	57	57	58	58	60	61	
	61	61	64	64	66	66	
	66	66	66	68	70	70	mean 59
On ES Day 90	57	66	61	54	69	75	
	47	44	54	55	62	55	
	59	56	56	60	69	56	
	51	67	57	65	51	65	
	63	58	69	67	54	57	mean 59
On ES Day 220	40	46	50	51	51	54	
	54	55	55	56	56	56	
	56	56	57	57	58	58	
	58	60	61	61	61	63	
	65	66	67	69	69	69	mean 58
On BA Day 220	74	74	74	78	80	80	
	80	82	82	83	83	83	
	84	84	84	84	85	86	
	86	89	89	90	90	93	
	94	98	100	102	102	void	mean 86

CLAIMS

1. A composition for preserving viable microorganisms, cells or tissue, the composition comprising:

   (a) a preservative combination of (i) trehalose (ii) a matrix protein bulking agent and (iii) a monosaccharide; and
   (b) a buffer.

2. A composition according to Claim 1 wherein the weight ratio of the trehalose: bulking agent is 5:1 to 0.5:1.

3. A composition according to Claims 1 or 2 wherein the weight ratio of the trehalose: bulking agent is 3.5:1 to 1.5:1.

4. A composition according to any preceding claim wherein the bulking agent is, or comprises, a protein of molecular weight 50-100KD.

5. A composition according to Claim 4 wherein the bulking agent is an albumin.

6. A composition according to any preceding claim, comprising:

   (i) 5 to 30 wt% trehalose;
   (ii) 1 to 10 wt% bulking agent; and
   (iii) 60 to 94 wt% aqueous buffer.

7. A composition according to any previous claim wherein the monosaccharide is a reducing sugar.

8. A composition according to Claim 7 wherein the reducing sugar is glucose.

9. A composition according to any of Claims 7-8 comprising 0.1 to 3 wt% monosaccharide.

10. A composition according to any preceding claim, further comprising a structural additive.

11. A composition according to Claim 10 wherein the structural additive is water-soluble.

12. A composition according to Claim 11 wherein the structural additive is, or comprises, a water-soluble polymeric carbohydrate.

13. A composition according to any of Claims 10-12 wherein the structural additive is carboxymethylcellulose or hydroxyalkylcellulose.

14. A composition according to any of Claims 10-13 wherein the structural additive and all other components are water-soluble so that when a dried composition of the invention is reconstituted in water it dissolves substantially completely leaving a clear solution.

15. A composition according to any preceding claim further comprising a colouring agent.

16. A composition according to any preceding claim wherein the microorganisms are selected from the group consisting of bacteria, viruses, protozoa and fungi.

17. A composition according to any preceding claim wherein the solids content is in the range of from 10-50wt%.

18. A process for preserving viable microorganisms, cells or tissue, comprising the steps of:

(a) combining the microorganisms, cells or tissue with the composition of any of Claims 1 to 17 to form a preserving preparation,;
(b) drying the preserving preparation at a drying temperature not below 0°C; and
(d) storing the dried preparation obtained.

19. A process according to Claim 18 wherein step (b) is carried out at atmospheric pressure.

20. A process according to Claim 18 or Claim 19 wherein step (b) is carried out by passing a draught of air, inert gas or a mixture thereof over the preserving preparation in the presence of a drying agent.

21. A process according to Claim 20 wherein step (b) comprises:

(i) partially drying the preserving preparation by passing a draught of air, inert gas or a mixture thereof over the preserving preparation in the presence of a drying agent; and

(ii) subjecting the partially dried preserving preparation to further drying at reduced temperature.

22. A process according to any of Claims 20-21 wherein the drying agent is silica gel.

23. A process according to any of Claims 18-22 comprising drying the preserving preparation at reduced pressure.

24. A process according to any of Claims 18-23 comprising depositing the preserving preparation onto a hydrophobic surface prior to drying.

25. A process according to Claim 24 wherein the volume of preserving preparation deposited onto the hydrophobic surface is in the range of from 5-100 µl.

26. A process according to any of Claims 18-25 wherein one or more of the steps (a) to (c) are carried out in a substantially oxygen-free environment.

27. A dried composition according to any of Claims 1-15 further comprising a viable microorganism and having a solids content of at least 80%.

28. A dried composition according to Claim 27 wherein the viable microorganisms are bacteria, or a mixture of bacteria of different strains, or a combination of bacteria and mammalian cells.

29. A dried composition according to any of Claims 27-28 having a defined microorganism count wherein said microorganism count remains substantially stable in storage at ambient temperature.

30. A dried composition according to any of Claims 27-29 wherein the composition is fully dispersible when reconstituted with water or aqueous buffer solution.

31. A method of preserving viable microorganisms, cells or tissue, comprising:-

(a) combining viable microorganisms, cells or tissue with a preserving solution comprising a composition according to Claim 1;

(b) drying the combination of (a) to form a dried preparation having a solids content of at least 80% by weight; and

(c) counting the viable microorganisms, cells or tissue in the dried preparation;

whereby the dried preparation can be combined with aqueous buffer to yield an aqueous preparation comprising a predetermined count of viable microorganisms, cells or tissue.

32. A method of preserving viable microorganisms, comprising drying an aqueous preparation of viable microorganisms in the presence of a composition according to Claim 1 to form a dried preparation, counting the viable microorganisms in the dried preparation to obtain a counted dried preparation, and storing the counted dried preparation, wherein the counted dried preparation when reconstituted with water or aqueous medium forms an aqueous preparation containing a predetermined number of viable microorganisms.

33. A method according to Claim 31 or 32 wherein the method comprises forming at least first and second dried preparations, counting the viable microorganisms, cells or tissue in the first dried preparation and thereby estimating the number of viable microorganisms, cells or tissue in the second dried preparation.

34. A method according to Claim 33 comprising forming a plurality of dried preparations, and counting a selected sample of those dried preparations so as to estimate the count of viable microorganisms, cells or tissue in the remaining dried preparations.

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WO 01/05941 A3

(54) Title: STORAGE OF MICROORGANISMS, CELLS AND TISSUE

(57) Abstract: A composition for preserving viable microorganisms, cells or tissue comprises (a) a preservative combination of (i) a non-reducing disaccharide and (ii) a bulking agent; and (b) a buffer. Also described is a method of preserving viable microorganisms, cells or tissue, comprising combining viable microorganisms, cells or tissue with a preserving solution comprising a non-reducing disaccharide, drying the combination to form a dried preparation having a solids content of at least 80 % by weight, and counting the viable microorganisms, cells or tissue in the dried preparation, whereby the dried preparation can be combined with aqueous buffer to yield an aqueous preparation comprising a predetermined count of viable microorganisms, cells or tissue.

Combined Declaration and Power of Attorney for Patent Application with Delegation

Docket Number: 1581.0890000/RWE/MTT

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled Storage of Microorganisms, Cells and Tissue , the specification of which is attached hereto unless the following box is checked:

☐ was filed on _____;
as United States Application Number or PCT International Application Number _____; and
was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application, which designated at least one country other than the United States listed below, and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed
9916790.0	United Kingdom	16 July 1999	☐ Yes ☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)	
_____	_____	_____	☐ Yes ☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

_____	_____
(Application No.)	(Filing Date)
_____	_____
(Application No.)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

PCT/GB00/02738	17 July 2000	Pending
(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)
_____	_____	_____
(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)

10031092 .071902

Appl. No.
Docket No. 1581.0890000/RWE/MTT

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

16

Robert G. Sterne, Esq., Reg. No. 28,912; Edward J. Kessler, Esq., Reg. No. 25,688; Jorge A. Goldstein, Esq., Reg. No. 29,021; Samuel L. Fox, Esq., Reg. No. 30,353; David K.S. Cornwell, Esq., Reg. No. 31,944; Robert W. Esmond, Esq., Reg. No. 32,893; Tracy-Gene G. Durkin, Esq., Reg. No. 32,831; Michele A. Cimbala, Esq., Reg. No. 33,851; Michael B. Ray, Esq., Reg. No. 33,997; Robert E. Sokohl, Esq., Reg. No. 36,013; Eric K. Steffe, Esq., Reg.No. 36,688; Michael Q. Lee, Esq., Reg. No. 35,239; Steven R. Ludwig, Esq., Reg. No. 36,203; Raz E. Fleshner, Esq., Reg. No. 34,331; John M. Covert, Esq., Reg. No. 38,759; and Linda E. Alcorn, Esq., Reg. No. 39,588.

I hereby authorize the U.S. attorneys named herein to accept and follow instructions from Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL, ENGLAND, as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication with the U.S. attorneys. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-0

Full name of sole or first inventor Anthony Arthur Codd	
Signature of sole or first inventor	Date 22/5/02
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Citizenship British	
Post Office Address Darras Hall, 213A Western Way, Ponteland, Northumberland NE20 9ND, United Kingdom	

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(Supply similar information and signature for subsequent joint inventors, if any)

POWER OF ATTORNEY FROM ASSIGNEE WITH DELEGATION

Public Health Laboratory Service Board , having a principal place of business at 61 Colindale Avenue, London NW9 5DF, United Kingdom , is assignee of the entire right, title, and interest for the United States of America (as defined in 35 U.S.C. §100), by reason of an Assignment to the Assignee executed on 22 May 2002 of an invention known as Storage of Microorganisms, Cells and Tissue (Attorney Docket No. 1581.0890000/RWE/MTT), which is disclosed and claimed in a patent application of the same title by the inventor(s) Anthony Arthur Codd (said application filed on _____ at the U.S. Patent and Trademark Office, having Application Number 10/031,062). RWE Reg No. 32,893

The Assignee hereby appoints the following U.S. attorneys to prosecute this application and any continuation, divisional, continuation-in-part, or reissue application thereof, and to transact all business in the U.S. Patent and Trademark Office connected therewith: Robert Greene Sterne, Registration No. 28,912; Edward J. Kessler, Registration No. 25,688; Jorge A. Goldstein, Registration No. 29,021; Samuel L. Fox, Registration No. 30,353; David K.S. Cornwell, Registration No. 31,944; Robert W. Esmond, Registration No. 32,893; Tracy-Gene G. Durkin, Registration No. 32,831; Michele A. Cimbala, Registration No. 33,851; Michael B. Ray, Registration No. 33,997; Robert E. Sokohl, Registration No. 36,013; Eric K. Steffe, Registration No. 36,688; Michael Q. Lee, Registration No. 35,239; Steven R. Ludwig, Registration No. 36,203; Raz E. Fleshner, Registration No. 34,331; John M. Covert, Registration No. 38,759; and Linda E. Alcorn, Registration No. 39,588. The Assignee hereby grants said attorneys the power to insert on this Power of Attorney any further identification that may be necessary or desirable in order to comply with the rules of the U.S. Patent and Trademark Office.

The Assignee hereby authorizes the U.S. attorneys named herein to accept and follow instructions from Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL, ENGLAND, as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and the Assignee. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by the Assignee.

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FOR: Public Health Laboratory Service Board

SIGNATURE: [signature]

BY: [illegible]

TITLE: Deputy Director of Public Service, PHLS

DATE: 23.6.02